Planta (2004) 218: 824–832
DOI 10.1007/s00425-003-1162-9

ORIGINAL ARTICLE

Ursula S. R. Röse · James H. Tumlinson

Volatiles released from cotton plants in response to *Helicoverpa zea* feeding damage on cotton flower buds

Received: 7 July 2003 / Accepted: 20 October 2003 / Published online: 19 November 2003

Abstract Feeding of *Helicoverpa zea* larvae on cotton (*Gossypium hirsutum* L.) flower buds (squares) for 24 or 48 h induced the release of a number of terpenes [(*E*)-β-ocimene, linalool, (*E*)-β-farnesene, (*E*,*E*)-α-farnesene, (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene], isomeric hexenyl butyrates, 2-methylbutyrates, indole and (*Z*)-3-hexenyl acetate. These compounds are not released in significant amounts from undamaged squares and freshly damaged squares. The release of inducible compounds was not limited to the damaged squares themselves. The compounds were also released systemically from the upper undamaged leaves of the same plant after 72 h. However, the composition of the blend of systemically released volatiles differed from the blend released by damaged squares. The compounds that were systemically released from undamaged leaves in response to feeding on the squares were (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*)-β-farnesene, (*E*,*E*)-α-farnesene, and indole. This study shows that insect damage inflicted to the reproductive parts of a plant causes a systemic emission of volatiles from its vegetative parts.

Keywords Flower bud · *Gossypium* · *Helicoverpa* · Herbivory · Semiochemical · Volatile

U. S. R. Röse (✉) · J. H. Tumlinson
Center for Medical, Agricultural, and Veterinary Entomology, U.S. Department of Agriculture, Agricultural Research Service, 1700 SW 23rd Drive, Gainesville, FL 32608, USA
E-mail: roese@ice.mpg.de
Fax: +49-3641-571302

Present address: U. S. R. Röse
Max-Planck Institut für Chemische Ökologie, Hans-Knöll-Str. 8, 07745 Jena, Germany

Present address: J. H. Tumlinson
Department of Entomology, The Pennsylvania State University, University Park, PA 16802, USA

Introduction

According to the optimal defense theory, the reproductive parts of a plant such as flowers and seeds should be highly defended against herbivory because these parts make a large contribution to reproductive fitness (McKey 1974; Zangerl and Rutledge 1996). In addition, the more apparent a plant part is, the more vulnerable it is to attack and thus the more antiherbivore defenses it should contain (Feeny 1976; Rhoades and Cates 1976; Rhoades 1979). Flowers often possess visual or olfactory cues to make them highly apparent to pollinators, but this may also increase their apparency to herbivores. To overcome this potential conflict, a defense mechanism to protect the flower from herbivory seems likely.

Antiherbivore defenses have been found in the flowers of numerous plant species. For example, the glucosinolate content of flowers of *Arabidopsis thaliana* is substantial and higher than that of most of the vegetative parts of the plant (Brown et al. 2003). The flower buds of *Gossypium hirsutum* contain high concentrations of tannin and gossypol that have been shown to have an effect on development and survival of several cotton pests (Sharma and Agarwal 1982). And, the nicotine content of *Nicotiana attenuata* flowers is increased after herbivory and mechanical damage (Euler and Baldwin 1996). Flowers also release volatiles, but these have been mainly studied in the context of pollinator attraction (Dobson 1994). Nevertheless, flowers may change their volatile profile after pollination in a way that further pollinators are repelled. For example, flowers of the orchid *Ophris sphegodes* can change their odor emission from a blend mimicking the volatile profile of receptive female bees in order to attract males of *Andrena nigroaenea* to pollinate by 'pseudocopulatory'' behavior, to the release of a repellent volatile profile similar to nonreceptive female bees after pollination has occurred (Schiestl et al. 1999; Schiestl and Ayasse 2001). The repellence of further pollinators may prevent damage to the developing seeds and increase the probability of

pollination for unpollinated neighboring flowers. However, a change in the composition of a volatile blend may not only be linked to direct defenses, but may also change the attractiveness of the blend to natural enemies of the herbivores that attack the plant (indirect defense). Vegetative plant parts like leaves are known to defend themselves by emitting volatile compounds in response to herbivore attack (Dicke et al. 1990; Turlings et al. 1990). This herbivore-induced release of volatiles benefits the plant by attracting natural enemies of the herbivores that feed on its foliage and benefits parasitoids and predators by guiding them to potential hosts or prey on the plant (Dicke and Sabelis 1988; Turlings et al. 1991, 1995; Röse et al. 1998). Recently, the defensive function of induced volatiles was shown in field experiments (De Moraes et al. 1998; Bernasconi et al. 2001; Kessler and Baldwin 2001). For example, herbivore-induced volatiles increase egg predation rates by a generalist predator and decrease lepidopteran oviposition rates on *Nicotiana attenuata* plants emitting herbivore-induced volatiles in the field (Kessler and Baldwin 2001).

Several studies have shown that the vegetative parts of cotton plants release herbivore-inducible volatiles, and much is known about the timing and synthesis of these compounds in leaves (Loughrin et al. 1994, 1995; McCall et al. 1994; Röse et al. 1996; Paré and Tumlinson 1997) and the effect of those volatiles on parasitoids (Röse et al. 1998). Given the differences in value of leaves compared to flower buds for reproductive fitness, we focus in this study on the inducibility of volatiles from flower buds in response to caterpillar damage. The volatile compounds released from caterpillar-damaged plants can be divided into constitutive compounds and inducible compounds. Constitutive compounds present in the plant are released from damaged leaves immediately after the beginning of feeding damage, or even after the plant is only damaged mechanically (Turlings et al. 1990; Röse et al. 1996). These early stages of plant damage are characterized by the release of "green leafy" volatiles like (*Z*)-3-hexenal, (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate and additional constitutive monoterpenes and sesquiterpenes that are stored in lysigenous glands (Elzen et al. 1985). The constitutive terpenes in cotton are mostly cyclic and include α-pinene, β-pinene, limonene, β-caryophyllene, α-humulene and the acyclic monoterpene β-myrcene. After several hours of herbivore damage or on the next day, leaves start to release additional compounds that appear to be specifically released in response to herbivore damage. These herbivore-inducible compounds in cotton are acyclic terpenoids [i.e. (*E*)-β-ocimene, linalool, (*E*)-β-farnesene, (*E*,*E*)-α-farnesene, (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene] and (*Z*)-3-hexenyl acetate, indole, isomeric hexenyl butyrates, and 2-methylbutyrates (Loughrin et al. 1994; McCall et al. 1994).

Herbivore-induced compounds are not only released at the site of herbivore damage, but also systemically from the entire cotton plant (Röse et al. 1996). After several days of feeding by *Spodoptera exigua* larvae on the lower leaves of cotton plants, the upper undamaged leaves of the same plant released (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*)-β-farnesene, (*E*,*E*)-α-farnesene, and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (Röse et al. 1996). Cotton plants that are damaged on the lower leaves by *Helicoverpa zea* larvae release similar compounds systemically, as do cotton plants damaged on the lower leaves by *S. exigua* larvae (Röse et al. 1998). However, in the field *H. zea* larvae prefer feeding on flower buds (squares) of cotton plants. Volatiles collected from air surrounding detached squares of several cotton genotypes grown in the field revealed the release of several monoterpenes (Chang et al. 1988). The most abundant monoterpenes released from those detached squares were α-pinene, β-pinene, β-myrcene, limonene, and β-ocimene. However, nothing is known about the effect of herbivory on the volatile release from flower buds.

The objective of our study was to determine how herbivory of *H. zea* larvae may change the volatile profile of flower buds in cotton. In addition to a local release of volatiles from the reproductive parts of the plant, we investigated whether damage to the reproductive structures (flower buds) caused a systemic release of volatiles from the vegetative plant parts (leaves) that may be linked to indirect defenses.

Materials and methods

Plants

Approximately 8-week-old cotton plants, *Gossypium hirsutum* L. (Malvaceae), cv. Deltapine acala 90 (Delta and Pine Land Company, Hollandala, MS, USA), with three squares on each plant were used in all experiments. Cotton was grown in a greenhouse in a mixture of compost, peat moss and vermiculite (metro-mix 300; Scotts-Sierra Horticultural Products Company, Marysville, OH, USA) with natural light, under Florida summer conditions (14 h light:10 h dark cycle, 85 ± 10% RH, and 35 ± 10°C). Each cotton plant was grown from seed planted in a 16-cm-diameter pot and fertilized once at the time of planting with a 3- to 4-month formulation of Osmocote 14-14-14 (N-P-K) controlled-release fertilizer (Scotts-Sierra Horticultural Products Company).

Lepidopteran larvae

Corn earworm, *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae) larvae were obtained from the USDA rearing facilities in Gainesville, Florida. Larvae were reared on an artificial diet, based on pinto beans, according to the method of King and Leppla (1984). To encourage immediate feeding of larvae after being caged on the squares, second- and third-instar larvae were starved for 12 h prior to the experiments. Each larva was confined in a separate cage that consisted of two halves of a modified Petri dish, as previously described (Röse et al. 1996), to define the area of feeding damage.

Volatile collection from damaged cotton squares

This experiment was conducted to determine the volatiles released from undamaged cotton squares compared to freshly damaged

squares, and squares damaged by *H. zea* larvae for 24 and 48 h. For fresh caterpillar damage (SQR-FRESH) and undamaged controls (SQR-CTRL), squares were removed from the plants immediately prior to the collection of volatiles and each square placed into a separate volatile collection chamber in the laboratory. For fresh damage, one third-instar larva was placed on each square and volatiles were collected subsequently from 12 p.m. to 3 p.m.. For 24-h damage (SQR-24) and 48-h damage (SQR-48), one second-instar *H. zea* was transferred to each cotton square that remained attached to the plant. The larva was confined to a square by a cage and allowed to feed continuously on the square for 24 or 48 h. Only one square was damaged on each plant and each experiment was repeated six times with separate plants. Immediately prior to volatile collection, squares were detached from the plants with a razor blade and placed in the laboratory volatile collection system modified after Turlings et al. (1991). The volatile collection chamber consisted of parallel glass chambers, each made of two separate parts. The first part of each glass chamber consisted of a 5-cm-long, 0.5-cm-outside-diameter (OD) inlet, which widened into a section (10 cm long, 3 cm OD), containing a glass frit to assure laminar airflow. The second part, containing the cotton square, was 15 cm long (3.8 cm OD) with a 5-cm-long (0.5 cm OD) outlet. Both parts of each glass chamber had fitting glass ball joints that were clamped together. A volatile collection trap (6 cm long, 0.5 cm OD) with 25 mg Super-Q (Alltech Assoc., Deerfield, IL, USA) as an adsorbent was connected to the 0.5-cm outlet with a brass Swagelock fitting containing Teflon ferules. To collect volatiles with this push–pull system, humidified air, purified by passing through an activated-charcoal filter, was blown through the glass chambers and exited through a volatile collection trap. To prevent excessive pressure within the chambers, the air was pulled through the collection traps by applying a vacuum downstream. An equal airflow of 600 ml min^{-1} through each chamber was controlled by flowmeters (Aalborg Instruments, Monsey, NY, USA) downstream of the collection filters.

Volatile collections from undamaged leaves of plants with damaged squares

This experiment was conducted to determine the volatiles released from upper undamaged leaves of cotton plants, damaged on the squares below by *H. zea* larvae (SQR-SYST), as compared to volatiles collected from upper leaves of undamaged control plants (CTRL-SYST). Each experiment was replicated four times with separate plants. To inflict damage on the squares, one second-instar *H. zea* was transferred to each of three cotton squares that remained attached to the plant. The larva was confined to a square by a cage and allowed to feed continuously on the square for 72 h. After 4 days, volatiles were collected from upper undamaged leaves of the damaged and the control plant, while the squares remained outside the collection system. Volatile samples were collected from 12 p.m. to 3 p.m.. To collect volatiles, the upper four leaves of each plant were enclosed in a volatile collection chamber, that was part of an automated volatile collection system previously described (Heath and Manukian 1994; Röse et al. 1996).

Purified air entered the system through the air diffuser inlet on top of the glass chamber at a controlled rate of 5 l min^{-1}. Volatile collector traps (150 mm long, 5 mm OD), containing 50 mg Super-Q as an adsorbent, were inserted in the side sampling ports located symmetrically around the base of the multiport guillotine base. Volatiles emitted from the upper portion of the cotton plant enclosed within the glass chamber were swept downward by the incoming pure laminar airflow. They were sampled at the bottom of the chamber by pulling air at a rate of 1 l min^{-1} through the volatile collection traps from a controlled vacuum source attached to each volatile collector trap from the automated volatile collection system. Thus, 20% of the air passed through the collector traps during the 3-h collection period. The remaining 80% excess air escaped through the opening at the bottom of the guillotine around the stem of the plant, loosely plugged with cotton balls to prevent any abrasion of the plant stem on the guillotine blades. This positive pressure venting provided a barrier against all ambient air and prevented volatiles from the lower, damaged part of the plant from entering into the collection chamber containing the upper undamaged part of the plant.

Analysis of volatiles

Volatiles were extracted from the collector traps by washing with 170 µl methylene chloride (capillary GC/GC–MS solvent, Burdick & Jackson, Muskegon, MI, USA) for traps containing 25 mg adsorbent and 200 µl for traps containing 50 mg adsorbent. Internal standards were added (600 ng each of *n*-octane and nonyl acetate in 60 µl methylene chloride) to the extract. Samples were analyzed by gas chromatography (GC) and GC–mass spectroscopy (GC–MS). Of each collection sample, 1 µl was injected in the splitless mode on a bonded methyl silicone fused silica capillary column in a Hewlett-Packard gas chromatograph (model 5890 II plus) equipped with an auto injector (model 6890), a split–splitless capillary injector system and flame ionization detector (Röse et al. 1996). Helium at a linear flow velocity of 20 cm s^{-1} was used as a carrier gas. The temperature of the column oven was maintained at 40°C for 3 min, and then programmed at 5°C min^{-1} to 220°C, which was maintained for 10 min. The injector temperature was set at 220°C, the detector temperature at 260°C. Data collection, storage and subsequent analysis were performed on a Perkin Elmer chromatographic data system.

To identify compounds, volatiles were analyzed by GC–MS with a Finnigan ITS-40 Magnum (ion-trap) mass spectrometer operated in electron impact and chemical ionization modes. For GC–MS the same fused silica capillary column and a DB5MS column (J&W Scientific, Folsom, CA, USA) were used with helium as a carrier gas, and for chemical ionization isobutane was used as reagent gas. Constituents of the plant volatiles were identified by comparison of mass spectra with spectra in the Environmental Protection Agency–National Institutes of Health data base, the Environmental Protection Agency–National Institute of Standards and Technology data base, and spectra obtained of authentic compounds. GC retention times of plant volatiles were also compared with GC retention times of those authentic compounds on the methyl silicone column, and the DB5MS column whenever they were available.

Statistical analyses

Data were analyzed with the statistic program SYSTAT (Systat, Evanston, IL, USA). Comparisons yielding a P-value ≤ 0.05 were considered to be statistically significant. Since the amounts of the various volatiles released per square or per four leaves frequently decreased below detectable limits, the assumption that these amounts are normally distributed is unreasonable. Therefore, differences in the amounts of volatiles released from SQR-CTRL, SQR-FRESH, SQR-24, and SQR-48 were analyzed nonparametrically. The Kruskal–Wallis one-way analysis of variance was used to determine the significance of differences in volatile amounts of each compound between five replicates of each treatment. Pairwise comparisons of volatile amounts between SQR-CTRL and SQR-FRESH, between SQR-FRESH and SQR-24, and between SQR-24 and SQR-48 were analyzed by the Mann–Whitney U test. The significance level was adjusted by the Dunn–Šidák method to $\alpha' = 0.0169$ [$\alpha' = 1-(1-\alpha)^{1/k}$; $\alpha' = 1-1(1-0.05)^{1/3} = 0.0169$].

Differences in the amounts of volatiles released per plant between SQR-SYST and CTRL-SYST leaves were analyzed nonparametrically. The Mann–Whitney U test was used to determine the significance of differences in volatile amounts between four replicates of both treatments. In keeping with the nonparametric analytic approach to the data, observed volatile amounts were summarized by the median and corresponding range (minimum–maximum) for each treatment. The Fischer's exact test was used to determine the significance of differences in the number of damaged

squares abscised from six plants after feeding of *H. zea* on three squares of each plant for 4 days compared to undamaged squares of six control plants without herbivores.

Results

Volatiles released from undamaged and herbivore-damaged cotton squares

Overall comparisons between undamaged cotton squares (SQR-CTRL), freshly damaged cotton squares (SQR-FRESH), squares damaged for 24 h (SQR-24), and squares damaged for 48 h (SQR-48) by *H. zea* larvae, showed significant differences in most of the mono-, homo- and sesquiterpenes, as well as in isomeric hexenyl butyrates, 2-methylbutyrates and indole (Table 1). SQR-CTRL released only very small amounts of volatiles (Table 1). Compared to SQR-CTRL, SQR-FRESH released significantly higher amounts of the ''green leafy'' volatile (*Z*)-3-hexenal, constitutive monoterpenes (i.e. α-pinene, β-pinene, myrcene, limonene), the inducible monoterpene (*E*)-β-ocimene, and constitutive sesquiterpenes (i.e. β-caryophyllene, α-humulene; Table 1).

However, after 24 h of continuous feeding of *H. zea* on the squares (SQR-24), the composition of the blend changed significantly. Cyclic terpenoids that were predominant in the volatile blend of freshly damaged tissue were released in lesser amounts after 24 h, whereas a number of inducible, acyclic terpenoids that are known to be synthesized de novo in response to herbivore feeding on leaves (Paré and Tumlinson 1997) were detected in large amounts (Fig. 1). The most predominant volatile compounds released in large amounts from SQR-24 were the inducible, acyclic monoterpenes (*E*)-β-ocimene and linalool, followed by the shikimic acid/tryptophan pathway-derived indole that is also synthesized de novo, and (*Z*)-3-hexenyl acetate. In addition, inducible, acyclic sesquiterpenes [i.e. (*E*)-β-farnesene, (*E*,*E*)-α-farnesene, nerolidol] and two homoterpenes [(E)-4,8-dimethyl-1,3,7-nonatriene, (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene] were released, that were not detected, or detected only in small amounts from CTRL squares and SQR-FRESH (Table 1).

Table 1 Composition of volatile blends collected between 12 p.m. and 3 p.m. [medians over five replications with range of values (minimum to maximum) shown in parentheses] from undamaged cotton (*Gossypium hirsutum*) squares (SQR-CTRL) compared to volatiles that were collected from squares freshly damaged by *Helicoverpa zea*larvae (SQR-FRESH) or squares that were fed upon by *H. zea* larvae for 24 h (SQR-24 h) or that were fed upon for 48 h (SQR-48 h). *n* Compound not detectable

Compound	Nanograms of compound emitted over 3 h per square[a]			
	SQR-CTRL	SQR-FRESH	SQR-24 h	SQR-48 h
(*Z*)-3-Hexenal[c]	n[b] (n)[d]	34 (8–126)	6 (3–17)	14 (8–113)
(*E*)-2-Hexenal[c]	n (n–2)	n (n–71)	2 (1–4)	3 (1–5)
(*Z*)-3-Hexenol[c]	37 (n–50)	1 (n–50)	16 (n–58)	n (n–2)
(*Z*)-3-Hexenyl acetate[i]	14[b] (6–17)	36 (11–90)[e]	397 (196–571)[f]	14 (7–132)
Hexyl acetate[i]	n[b] (n)	n (n–6)[e]	47 (31–86)	35 (8–59)
(*Z*)-3-Hexenyl isobutyrate[i]	n[b] (n)	n (n)[e]	28 (9–43)	7 (n–14)
(*Z*)-3-Hexenyl butyrate[i]	n[b] (n)	n (n)[e]	55 (9–123)	16 (n–18)
(*E*)-2-Hexenyl butyrate[i]	n[b] (n)	n (n)[e]	60 (26–95)	18 (5–70)
(*Z*)-3-Hexenyl-2-methylbutyrate[i]	n[b] (n)	n (n)[e]	134 (41–172)	14 (6–26)
(*E*)-2-Hexenyl-2-methylbutyrate[i]	n[b] (n)	n (n–1)[e]	308 (178–471)[f]	166 (51–187)
(*Z*)-Jasmone[i]	n[b] (n)	n (n)	2 (n–35)	10 (3–49)
Indole[i]	n[b] (n)	n (n)[e]	466 (136–975)	162 (51–820)
α-Pinene[c]	20[b] (8–30)[d]	318 (199–2,007)[e]	9 (3–207)	5 (2–52)
β-Pinene[c]	5[b] (1–20)[d]	56 (33–327)[e]	5 (2–42)	3 (n–11)
Myrcene[c]	7[b] (4–19)[d]	183 (50–1,192)[e]	38 (28–111)	26 (18–66)
Limonene[c]	n[b] (n–1)[d]	23 (12–131)[e]	5 (4–22)	4 (4–8)
(*E*)-β-Ocimene[i]	4[b] (2–19)[d]	87 (17–781)[e]	1,418 (966–2,505)[f]	591 (253–836)
Linalool[i]	1[b] (n-3)	4 (2–16)[e]	697 (346–1,466)	453 (281–965)
(*E*)-4,8-Dimethyl-1,3,7-nonatriene[i]	n[b] (n)	n (n–2)[e]	355 (321–488)[f]	164 (49–262)
β-Caryophyllene[c]	14[b] (3–42)[d]	84 (66–622)	20 (3–146)	2 (n–34)
α-Humulene[c]	3[b] (n–7)[d]	23 (17–164)	9 (3–57)	n (n–10)
Unknown sesquiterpene hydrocarbon[c]	n[b] (n–29)[d]	59 (50–533)	18 (3–207)	2 (n–29)
(*E*)-β-Farnesene[i]	n[b] (n)	n (n–17)	11 (4–19)	11 (4–20)
(*E*,*E*)-α-Farnesene[i]	n[b] (n)	n (n)[e]	89 (84–166)[f]	27 (11–62)
Nerolidol[i]	n[b] (n)	n (n–57)	23 (10–32)	4 (n–20)
(*E*,*E*)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene[i]	n[b] (n)	n (n–3)[e]	24 (11–58)	9 (2–65)

[a]Observed volatile amounts are summarized by the median and corresponding range (minimum–maximum) for each treatment

[b]The Kruskal–Wallis one-way analysis of variance was used to determine the significance of differences in volatile amounts of each compound between five replicates of each treatment. These comparisons yielded a P value < 0.05 and were considered to be statistically significant

[c]Constitutive compounds

[d,e,f]Pairwise comparisons of volatile amounts between SQR-CTRL and SQR-FRESH ([d]), between SQR-FRESH and SQR-24 ([e]), and between SQR-24 and SQR-48 ([f]) were analyzed by the Mann–Whitney U test. The significance level was adjusted by the Dunn–Šidák method to $\alpha' = 0.0169$, $[\alpha' = 1-(1-\alpha)^{1/k}]$

[i]Induced compounds

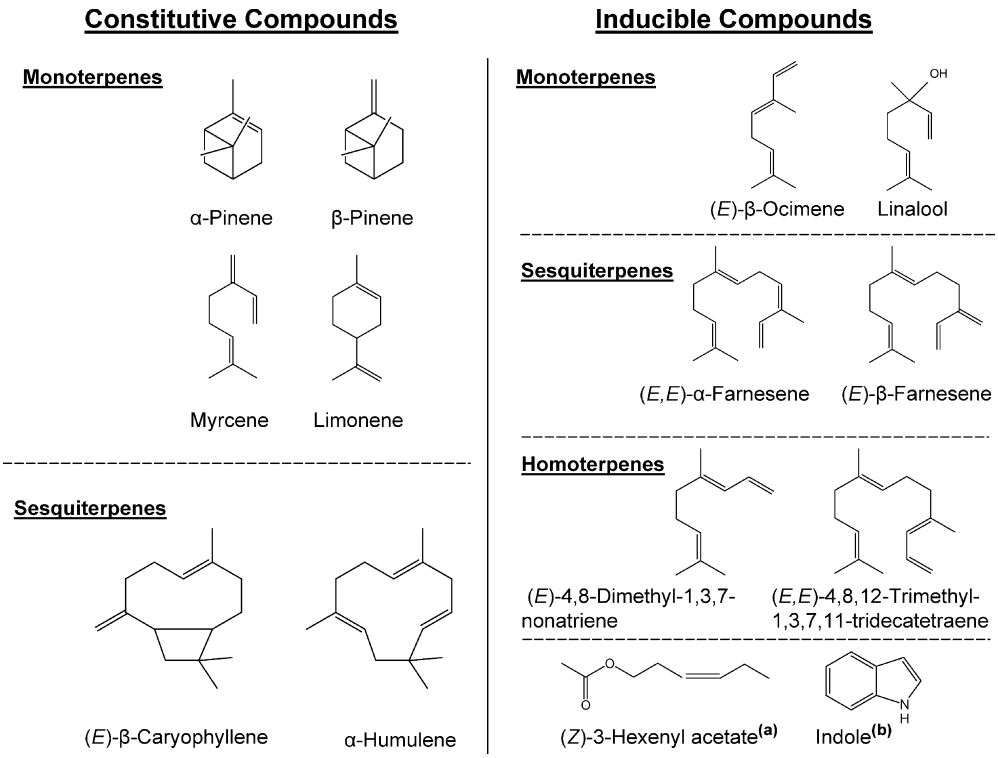

Fig. 1 Chemical structures of the major constitutive and inducible volatile compounds released from flower buds of cotton. *(a)* Compound synthesized via the lipoxigenase pathway; *(b)* compound synthesized via the shikimic acid/tryptophan pathway

Furthermore, a number of hexenyl butyrates and 2-methylbutyrates and (*Z*)-jasmone were detected that were not released from SQR-CTRL or SQR-FRESH. Squares that were damaged by *H. zea* for 48 h (SQR-48), released qualitatively the same volatile compounds as SQR-24. However, some of the inducible compounds [i.e. (*Z*)-3-hexenyl acetate, (*E*)-*β*-ocimene, (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*Z*)-3-hexenyl isobutyrate, (*Z*)-3-hexenyl-2-methylbutyrate, (*E*,*E*)-α-farnesene, nerolidol] were released in smaller amounts compared to volatiles released from SQR-24.

Volatile collections for more than 48 h were in most cases not possible, because feeding of *H. zea* on the squares resulted in abscission of the squares after 4 days (72 h). Of a total of 18 squares that were fed upon by the larvae, 88.8% (=16 squares) were abscised after 72 h compared to control squares that showed no abscission ($n=6$; $P=0.002$).

Systemic release of volatiles

On day 4 the upper undamaged leaves of plants with squares on which *H. zea* larvae had fed for 4 days (SQR-SYST) and control plants (CTRL-SYST) released myrcene, (*Z*)-3-hexenyl acetate, (*E*)-*β*-ocimene, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, indole, (*E*)-*β*-farnesene, (*E*,*E*)-α-farnesene, and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (Fig. 2). Feeding of *H. zea* larvae on squares induced the systemic release of significantly higher amounts of (*Z*)-3-hexenyl acetate, the monoterpenes (*E*)-*β*-ocimene and linalool, the homoterpene (*E*)-4,8-dimethyl-1,3,7-nonatriene, and the sesquiterpenes (*E*)-*β*-farnesene and (*E*,*E*)-α-farnesene, from undamaged leaves of the damaged plant (SQR-SYST) compared to leaves of control plants (CTRL-SYST; Table 2).

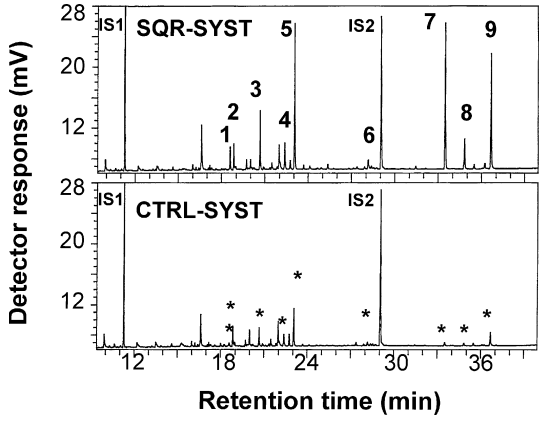

Fig. 2 Chromatographic profiles after analysis on the methyl silicone capillary column of systemically released volatiles from the upper undamaged leaves of a cotton (*Gossypium hirsutum*) plant damaged by corn earworms (*Helicoverpa zea*) on the squares (SQR-SYST) and from control leaves (CTRL-SYST) collected from 12 p.m. to 3 p.m. on day 4. *Compounds*: *1*, myrcene; *2*, (*Z*)-3-hexenyl acetate; *3*, (*E*)-*β*-ocimene; *4*, linalool; *5*, (*E*)-4,8-dimethyl-1,3,7-nonatriene; *6*, indole; *7*, (*E*)-*β*-farnesene; *8*, (*E*,*E*)-α-farnesene; *9*, (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. Added reference compounds were *n*-octane (*IS1*) and nonyl acetate (*IS2*). Peak numbers are the same as in Table 2. *Asterisks* (*) indicate compounds in the *lower* chromatogram that align with, and are the same as, those in the *upper* chromatogram

Table 2 Composition of volatile blends collected on day 4 from 12 p.m. to 3 p.m. [medians over four replications with range of values (minimum to maximum) shown in parenthesis] from the four upper undamaged leaves of cotton plants with *H. zea* damaged squares (SYST-SQR) and of undamaged control plants (CTRL-SYST). *n* Compound not detectable

Peak	Compound	Nanograms of compound emitted over 3 h per four leaves[a]	
		SQR-SYST	CTRL-SYST
1	Myrcene	370 (110–1,450)	170 (35–325)
2	(*Z*)-3-Hexenyl acetate	1,125[b] (45–3,515)	390 (55–440)
3	(*E*)-β-Ocimene	805[b] (400–1,095)	155 (135–335)
4	Linalool	405[b] (275–535)	120 (65–260)
5	(*E*)-4,8-Dimethyl-1,3,7-nonatriene	1,675[b] (635–2,760)	420 (100–690)
6	Indole	200[b] (25–360)	10 (n–85)
7	(*E*)-β-Farnesene	2,665[b] (730–3,395)	45 (15–75)
8	(*E*,*E*)-α-Farnesene	185[b] (65–635)	20 (10–50)
9	(*E*,*E*)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene	1,530 (75–2,675)	275 (50–300)

[a]Observed volatile amounts are summarized by the median and corresponding range (minimum–maximum) for each treatment
[b]Differences in volatile amounts between four SQR-SYST and four CTRL-SYST replicates are significant at $P \leq 0.05$, as determined by the Mann–Whitney U test

Discussion

After herbivory, the composition of the blend of volatiles released from different organs of a cotton plant, such as flower buds (in this study), leaves (McCall et al. 1994; Loughrin et al. 1994; Röse et al. 1996), or damaged bolls (fruit) and flowers (Turlings et al. 1993) may vary. In our study, damaged squares released considerably less of the green leafy volatiles [(*Z*)-3-hexenal, (*E*)-2-hexenal, (*Z*)-3-hexenol] compared to what has been reported to be released from damaged leaves (Loughrin et al. 1994). The green leafy volatile (*Z*)-3-hexenyl acetate, which has not been found in damaged flowers and bolls (Turlings et al. 1993), was detected from damaged squares in our experiments and has been reported to be released from damaged leaves (Loughrin et al. 1994).

The volatile blend released from squares damaged for 24 h and 48 h was mainly composed of (*E*)-β-ocimene, linalool, indole, (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*)-2-hexenyl-2-methylbutyrate and (*Z*)-3-hexenyl acetate. Inducible acyclic sesquiterpenes like (*E*)-β-farnesene, and (*E*,*E*)-α-farnesene that are released in larger quantities from leaves damaged by herbivores for 48 h (Loughrin et al. 1994), were only released in small amounts from squares. In addition, damaged squares also released (*Z*)-3-hexenyl isobutyrate. Squares that were damaged for 48 h released smaller amounts of volatiles compared to squares with 24 h of damage. This may be explained by an abscission of squares in response to 72 h feeding which may be preceded by a reduced volatile production. Abscission of squares (Holman and Oosterhuis 1999) and of bolls (Gore et al. 2000) in response to larval feeding has been described. The abscission of bolls 7 days after anthesis of up to 50% by 72 h after infestation with *H. zea* (Gore et al. 2000) is lower compared to 88% abscission of squares after 72 h of *H. zea* feeding in our experiments. The abscission of squares may be the most efficient way for the plant to prevent further investment of resources in a flower tissue that can no longer be functional in reproduction after severe damage.

The acyclic monoterpenes (*E*)-β-ocimene and linalool are often reported as flower volatiles (Joulain 1987). These compounds, which were released in large amounts from squares damaged by herbivores for 24 h, can be detected by a variety of insects, including adult Lepidoptera (Raguso et al. 1996), aphids (Quiroz and Niemeyer 1998), honey bees (Henning et al. 1992), predators (Weissbecker et al. 1999), and parasitic wasps (Du YongJun et al. 1998), and may serve as attractants (Du YongJun et al. 1998) to guide insects to a host plant or potential prey. For example the aphid parasitoid *Aphidius ervi* was able to respond to (*E*)-β-ocimene and linalool in electroantennograms (EAGs) and was attracted to those compounds in flight-tunnel experiments (Du YongJun et al. 1998). Also, females of the parasitic wasps *Microplitis rufiventris* and *Cotesia marginiventris* that parasitize *H. zea* larvae respond to (*E*)-β-ocimene and linalool in EAGs (Dr. S. Gouinguenené, Eidg. Forschungsanstalt für Obst-, Wein, und Gartenbau, Wädenswil, Switzerland; personal communication).

Flower volatiles and volatiles from vegetative parts of a plant can be exploited by herbivores to locate a food source or a suitable plant for egg deposition (Minyard et al. 1969). Volatiles released from uninjured squares or leaf tissue may attract female moths in search of an oviposition site (Tingle and Mitchell 1992). Adult females of *H. zea* do not lay their eggs in clusters but instead eggs are individually placed on host plant tissue. Upon hatching, larvae search for a suitable feeding site, usually preferring the reproductive structures of a plant. As larvae mature, they become very aggressive and cannibalistic. To avoid competition, female moths searching for an oviposition site may prefer undamaged plants with no herbivores present. An indicator of whether a plant is already occupied by other herbivores may be conveyed by volatiles released from

herbivore-damaged plant tissue. Because *H. zea* larvae prefer feeding on reproductive structures, the volatiles released from herbivore-damaged flower buds may be a cue for the presence of competing herbivores and may lead to avoidance of those plants by egg-laying moths. For example mated females of the cabbage looper, *Trichoplusia ni*, were attracted to cotton plants damaged by caterpillars for 20 h, whereas the attractiveness of cabbage plants to a moth is decreased after damage (Landolt 1993). Interestingly, for oviposition these moths preferred undamaged cotton and cabbage plants. Furthermore, tobacco plants that emit herbivore-induced volatiles at night are deterrent to female moths of *Heliothis virescens* (De Moraes et al. 2001). The compounds that were found to be exclusively released at night from *H. virescens*-damaged tobacco plants and that could explain the moth repellence included (*Z*)-3-hexenyl butyrate, (*Z*)-3-hexenyl isobutyrate, (*Z*)-3-hexenyl acetate, (*Z*)-3-hexenyl tiglate and an unidentified compound. Herbivore-damaged cotton squares in our experiments released three of those compounds: (*Z*)-3-hexenyl butyrate, (*Z*)-3-hexenyl isobutyrate, and (*Z*)-3-hexenyl acetate. All of these three compounds were induced specifically in response to 24 h of feeding on the squares. Like in tobacco plants, these compounds may have a deterrent effect on conspecific female moths in search for an oviposition site.

The release of herbivore-inducible volatiles was not limited to the damaged squares. Compared to undamaged leaves of control plants (CTRL-SYST), feeding by *H. zea* larvae on squares induced a systemic release of significantly higher amounts of (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, indole, (*E*)-β-farnesene, and (*E*,*E*)-α-farnesene from SQR-SYST leaves. All of these compounds can also be detected from undamaged cotton leaves in response to feeding of corn earworm larvae and beet armyworm larvae on the lower leaves (Röse et al. 1998). Therefore, cotton plants respond to caterpillar damage on squares or leaves with a systemic release of similar volatiles. However, we observed some variation in the amounts of systemically released compounds like β-pinene, myrcene, and indole. This variation may be due to differences in the developmental stage of the cotton plants, which were 3 weeks older in our experiments than plants used to observe a systemic volatile release in response to feeding on leaves (Röse et al. 1996). Seasonal effects on the amount of total volatiles collected were reported for cotton plants growing in the field, where the amounts of total volatiles collected reached a maximum when the cotton plant squaring and flowering was highest (Hedin 1976). Furthermore, volatile monoterpenes collected from squares fluctuate with the age of the cotton plant (Chang et al. 1988). A study on young and old cucumber leaves reported that the age of plants may affect the composition of volatiles released (Takabayashi et al. 1994).

In previous experiments, we showed that females of the specialist parasitic wasp *M. croceipes* and of the generalist parasitic wasp *Cotesia marginiventris* were highly attracted to systemically released volatiles in response to beet armyworm feeding on the lower leaves (Röse et al. 1998). However, the specialist *M. croceipes* does not parasitize beet armyworm larvae, but attacks corn earworm larvae as hosts, which prefer to feed on plant terminals and squares. The fact that volatile compounds systemically released in response to feeding of corn earworm larvae on squares were similar to those systemically released in response to a non-host feeding on leaves may explain why the wasps are attracted. It appears that cotton plants do not only respond with a similar systemic release of volatiles after feeding damage by different herbivore species, but that feeding of larvae on leaves and squares induces a similar systemic response of the plant from undamaged leaves. Subtle differences in the blend may be learned by the wasps through experience. Cotton plants that release inducible volatile compounds systemically are innately attractive to parasitoids of a generalist and a specialist parasitoid species (Röse et al. 1998). This clearly shows an active role of the plant in recruiting beneficial insects to damaged plants. The systemic signaling from reproductive parts of the plant to the vegetative parts of the plant may increase the overall detectability of the damaged plant for predators and parasitoids. More specific information about a potential host or non-host feeding on the plant may be provided by the plant tissue (e.g. squares, flowers, leaves) that is damaged, directly from the feeding site (De Moraes et al. 1998) or from volatiles emitted by host-products like frass (Röse et al. 1997). Volatiles released from herbivore-damaged squares may function as repellents of cotton pests as well as attractants for parasitoids and predators. As flower buds are rather apparent plant parts with a high value for reproductive fitness, it may be suggested that these should be defended constitutively to avoid the delay inherent in forming herbivore-induced defenses (McKey 1979; Zangerl and Rutledge 1996). However, here we show evidence for an inducible local and systemic release of volatiles from flower buds that may mediate direct and indirect defenses.

In summary, undamaged cotton squares in this study like undamaged cotton leaves in previous studies released only very small amounts of volatiles. Herbivory on cotton squares induced the release of compounds similar to those detected from herbivore-damaged cotton leaves after 24–48 h of herbivory, but with quantitative differences in the composition of the blend. Furthermore, feeding on squares induced a systemic release of volatiles that was similar to the volatiles systemically detected in response to feeding on leaves. However, the blend of systemically released volatiles differed quantitatively.

Acknowledgements We are grateful to Ted Turlings (Institute of Zoology, University of Neuchatel, Switzerland), Jonathan Gershenzon and Ian Baldwin (MPI for Chemical Ecology, Jena, Germany) and Yasmin J. Cardoza (CSIRO Entomology, Wembley,

Australia) for their helpful comments concerning the initial manuscript. We thank the Delta and Pine Land Company (Hollandala, MS, USA) for donation of the seeds.

References

Bernasconi Ockroy ML, Turlings TCJ, Edwards PJ, Fritzsche-Hoballah ME, Ambrosetti L, Bassetti P, Dorn S (2001) Response of natural populations of predators and parasitoids to artificially induced volatile emissions in maize plants (*Zea mays* L.). Agric For Entomol 3:201–209

Brown PD, Tokuhisa JG, Reichelt M, Gershenzon J (2003) Variation of glucosinolate accumulation among different organs and developmental stages of *Arabidopsis thaliana*. Phytochemistry 62:471–481

Chang JF, Benedict JH, Payne TL, Camp BJ (1988) Volatile monoterpenes collected from air surrounding flower buds of seven cotton genotypes. Crop Sci 28:685–688

De Moraes CM, Lewis WJ, Paré PW, Alborn HT, Tumlinson JH (1998) Herbivore-infested plants selectively attract parasitoids. Nature 393:570–773

De Moraes CM, Mescher MC, Tumlinson JH (2001) Caterpillar-induced nocturnal plant volatiles repel conspecific females. Nature 410:577–580

Dicke M, Sabelis MW (1988) How plants obtain predatory mites as bodyguards. Neth J Zool 38:148–165

Dicke M, van Beek TA, Posthumus MA, Ben Dom N, van Bokhoven H, de Groot AE (1990) Isolation and identification of volatile kairomone that affects acarine predator–prey interactions. J Chem Ecol 16:381–396

Dobson HEM (1994) Floral volatiles in insect biology. In: Bernays EA (ed) Insect–plant interactions, vol 5. CRC, Boca Raton, pp 47–81

Du YongJun, Poppy GM, Powell W, Pickett JA, Wadhams LJ, Woodcock CM (1998) Identification of semiochemicals released during aphid feeding that attract parasitoid *Aphidius ervi*. J Chem Ecol 24:1355–1368

Elzen GW, Williams HJ, Bell AA, Stipanovic RD, Vinson SB (1985) Quantification of volatile terpenes of glanded and glandless *Gossypium hirsutum* L. cultivars and lines by gas chromatography. J Agric Food Chem 33:1079–1082

Euler M, Baldwin IT (1996) The chemistry of defense and apparency in the corollas of *Nicotiana attenuata*. Oecologia 107:102–112

Feeny PP (1976) Plant apparency and chemical defense. In: Wallace JW, Mansell RL (eds) Biochemical interaction between plants and insects. Recent advances in phytochemistry, vol 10. Plenum, New York, pp 1–40

Gore J, Leonard BR, Church GE, Russell JS, Hall TS (2000) Cotton boll abscission and yield losses associated with first-instar bollworm (Lepidoptera: Noctuidae) injury to non-transgenic and transgenic Bt cotton. J Econ Entomol 93:690–696

Heath RR, Manukian A (1994) An automated system for use in collecting volatile chemicals released from plants. J Chem Ecol 20:593–608

Hedin PA (1976) Seasonal variations in the emission of volatiles by cotton plants growing in the field. Environ Entomol 5:1234–1238

Henning JA, Peng YS, Montague MA, Teuber LR (1992) Honeybee (Hymenoptera, Apidae) behavioral-response to primary alfalfa (Rosales, Fabaceae) floral volatiles. J Econ Entomol 85:233–239

Holman EM, Oosterhuis DM (1999) Cotton photosynthesis and carbon partitioning in response to floral bud loss due to insect damage. Crop Sci 39:1347–1351

Joulain D (1987) The composition of the headspace from fragrant flowers: further results. Flav Frag J 2:149–155

Kessler A, Baldwin IT (2001) Defensive function of herbivore-induced plant volatile emission in nature. Science 291:2141–2144

King EG, Leppla NC (1984) Advances and challenges in insect rearing. Agriculture Research Service USDA, U.S. Government Printing Office, Washington, D.C.

Landolt PJ (1993) Effects of host plant leaf damage on cabbage looper moth attraction and oviposition. Entomol Exp Appl 67:79–85

Loughrin JH, Manukian A, Heath RR, Turlings TCJ, Tumlinson JH (1994) Diurnal cycle of emission of induced volatile terpenoids by herbivore-injured cotton plants. Proc Natl Acad Sci USA 91:11836–11840

Loughrin JH, Manukian A, Heath RR,Tumlinson JH (1995) Volatiles emitted by different cotton varieties damaged by feeding beet armyworm larvae. J Chem Ecol 21:1217–1227

McCall PJ, Turlings TCJ, Loughrin J, Proveaux AT, Tumlinson JH (1994) Herbivore-induced volatile emissions from cotton (*Gossypium hirsutum* L.) seedlings. J Chem Ecol 20:3039–3050

McKey D (1974) Adaptive patterns in alkaloid physiology. Am Nat 108:305–320

McKey D (1979) The distribution of secondary compounds within plants. In: Rosenthal GA, Janzen DH (eds) Herbivores. Their interaction with secondary plant metabolites. Academic Press, New York, pp 56–133

Minyard JP, Hardee DD, Gueldner RC, Thompson AC, Wiygul G, Hedin PA (1969) Constituents of the cotton bud. Compounds attractive to the boll weevil. J Agr Food Chem 17:1093–1097

Paré PW, Tumlinson JH (1997) Induced synthesis of plant volatiles. Nature 385:30–31

Quiroz A, Niemeyer HM (1998) Olfactometer-assessed responses of aphid *Rhopalosiphum padi* to wheat and oat volatiles. J Chem Ecol 24:113–124

Raguso RA, Light DM, Pickersky E (1996) Electroantennogram responses of *Hyles lineata* (Sphingidae: Lepidoptera) to volatile compounds from *Clarkia breweri* (Onagraceae) and other moth-pollinated flowers. J Chem Ecol 22:1735–1766

Rhoades DF (1979) Evolution of plant chemical defense against herbivores. In: Rosenthal GA, Janzen DH (eds) Herbivores: their interaction with secondary plant metabolites, 2nd edn. Academic Press, New York, pp 3–54

Rhoades DF, Cates RG (1976) Toward a general theory of plant antiherbivore chemistry. In: Wallace JW, Mansell RL (eds) Biochemical interaction between plants and insects. Recent advances in phytochemistry, vol 10. Plenum, New York, pp 168–213

Röse USR, Manukian A, Heath RR, Tumlinson JH (1996) Volatile semiochemicals released from undamaged cotton leaves: a systemic response of living plants to caterpillar damage. Plant Physiol 111:487–495

Röse USR, Alborn HT, Makranczy G, Lewis WJ, Tumlinson JH (1997) Host recognition by the specialist endoparasitoid *Microplitis croceipes* (Hymenoptera: Braconidae): role of host and plant related volatiles. J Insect Behav 10:313–330

Röse USR, Lewis WJ, Tumlinson JH (1998) Specificity of systemically released cotton volatiles as attractants for specialist and generalist parasitic wasps. J Chem Ecol 24:303–319

Sharma HC, Agarwal RA (1982) Effect of some antibiotic compounds in *Gossypium* on the post-embryonic development of spotted bollworm (*Earias vittella*). Entomol Exp Appl 31:225–228

Schiestl FP, Ayasse M (2001) Post-pollination emission of a repellent compound in a sexually deceptive orchid: a new mechanism for maximising reproductive success? Oecologia 126:531–534

Schiestl FP, Ayasse M, Paulus HF, Lofstedt C, Hansson BS, Ibarra F, Franke W (1999) Orchid pollination by sexual swindle. Nature 399:421–422

Takabayashi J, Dicke M, Takahashi S, Posthumus MA, Van Beek TA (1994) Leaf age affects composition of herbivore-induced synomones and attraction of predatory mites. J Chem Ecol 20:373–386

Tingle FC, Mitchell ER (1992) Attraction of *Heliothis virescens* (F.) (Lepidoptera: Noctuidae) to volatiles from extracts of cotton flowers. J Chem Ecol 18:907–914

Turlings TCJ, Tumlinson JH, Lewis WJ (1990) Exploitation of herbivore-induced plant odors by host seeking parasitic wasps. Science 250:1251–1253

Turlings TCJ, Tumlinson JH, Eller FJ, Lewis WJ (1991) Larval-damaged plants: source of volatile synomones that guide the parasitoid *Cotesia marginiventris* to the micro-habitat of its hosts. Entomol Exp Appl 58:75–82

Turlings TCJ, Wäckers FL, Vet LEM, Lewis WJ, Tumlinson JH (1993) Learning of host-finding cues by hymenopterous parasitoids. In: Papaj DR, Lewis AC (eds) Insect learning. Chapman & Hall, New York, pp 51–78

Turlings TCJ, Loughrin JH, McCall PJ, Röse USR, Lewis WJ, Tumlinson JH (1995) How caterpillar-damaged plants protect themselves by attracting parasitic wasps. Proc Natl Acad Sci USA 92:4169–4174

Weissbecker B, Van Loon JJA, Dicke M (1999) Electroantennogram responses of a predator, *Perillus bioculatus*, and its prey, *Leptinotarsa decemlineata*, to plant volatiles. J Chem Ecol 25:2313–2325

Zangerl AR, Rutledge CE (1996) The probability of attack and patterns of constitutive and induced defense: a test of optimal defense theory. Am Nat 147:599–608